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VARIATIONS OF LISTERIA MONOCYTOGENES PRODUCED BY BETA PARTICLES FROM RADIOPHOSPHORUS

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One of the most widely used methods to induce variations in micro-organisms is the application of various types of radiation. Ultraviolet light and X-rays have been used most frequently for this purpose. While beta particles have previously been used to irradiate bacteria, they have usually been employed to determine the lethal effects. Beta radiations were obtained from cathode ray tubes or from some naturally radioactive substance which also emitted alpha particles or gamma rays. Recently the radioactive isotope of phosphorus, commonly referred to as P^{32}, which emits only beta particles has been made available. One of the advantages of using this source of beta particles is that the radiophosphorus, in the form of a phosphate, can be dissolved and distributed uniformly throughout the medium. This minimizes the absorption of the beta particles by materials other than the bacterial cell substance and the medium, thus making possible the more accurate estimate of the amount of radiation and the number of cells which are affected by it.

Although von Schroetter (1927) observed some interesting morphological changes while studying the action of radium on microorganisms over twenty years ago, the use of ionizing radiations, other than X-rays, to induce variations in microorganisms has largely been limited to the past ten years. Whelden (1940) demonstrated variant production in bacteria by low voltage cathode rays. Neutrons were shown to induce variation in *Penicillium notatum* (Myers and Hanson, 1945: Hanson, Myers, Stahly and Birkeland, 1946) and beta radiations from radiophosphorus were shown to produce variations in *Neurospora crassa* (Giles and Lederberg, 1948). Morphological and physiological variations of *Bacillus globigii* induced by beta particle irradiation were reported previously (Hunter, Stahly and Myers, 1948). In the present study, the effects of ionizing radiations on the morphologic, biochemical, immunogenic, and pathogenic characteristics of *Listeria monocytogenes* were investigated.

MATERIALS AND METHODS

The Ohio State University stock strain 144 of *Listeria monocytogenes* was selected as the test organism. This strain formed small, convex, grayish-white colonies with a regular periphery and produced a small zone of beta hemolysis on horse blood agar. Less than 1 per cent of the colonies appeared slightly larger and rougher than the rest. A smooth colony was picked and restreaked; no more rough colonies appeared during serial transfer on five plates. A colony, picked from the fifth plate, was inoculated upon a blood agar slant and was kept as the stock culture.

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The radiophosphorus used in these experiments was obtained from the Atomic Energy Commission at Oak Ridge, Tennessee, in the form of potassium dihydrogen phosphate with a specific activity of approximately two P^{32} atoms per ten million phosphorus atoms. A 2 per cent solution of the phosphate was prepared at the Ohio State University Medical Center. A control solution which contained the same concentration of non-radioactive potassium dihydrogen phosphate was prepared also. The amount of standard sodium hydroxide required to adjust the pH of the control solution to 7.0 was determined; this amount of the same base was also added to the radioactive solution to adjust it to the same pH. Both of these solutions were sterilized by autoclaving for 20 minutes at 15 pounds pressure.

The organism was grown on a blood agar slope for 24 hours and then the cells were suspended in 5 ml. of doubly distilled water. Both the exposure tube and the control tube were prepared by transferring 1 ml. of the bacterial suspension, by means of an automatic pipette, into 10 ml. of each of the phosphate solutions. At the time of inoculation the radiophosphate solution had an activity of 120 microcuries per ml. Application of the formula of Marinelli et al (1948) modified by an estimate of the amount of beta ray absorption by the test tube, resulted in a calculated value of approximately 45,000 equivalent roentgen units to which the culture was exposed during 11 days.

At 24-hour intervals samples were withdrawn from both the exposure tube and the control tube and streaked over a series of beef-infusion horse-blood agar plates. These plates were examined after 48 hours of incubation at 37 C. and any colony which appeared different from the stock colonies was picked and streaked upon a medium of the same composition. On the plates streaked from the control tube no colonies were detected which differed from the normal colonies of the parent strain. From the exposure tube twenty-one colonies were selected which differed from the parent strain in some morphological characteristic discernible under the stereoscopic microscope. One of these strains (number 4) was lost during subculture procedures. The other twenty strains were examined with respect to their morphologic, antigenic, physiologic and pathogenic characteristics.

EXPERIMENTAL RESULTS

Morphological Characteristics

The twenty strains which were isolated differed from the parent strain and generally from each other in one or more of the following characteristics: size, opacity, elevation, color, texture, roughness, and degree of hemolysis on blood agar of the colonies and the size, shape and staining reactions of the cells.

Agglutination and precipitation reactions

An antiserum was prepared against the unirradiated parent strain by the procedures recommended by Paterson (1940) for the demonstration of a somatic antigen common to all types of *Listeria monocytogenes.* Cell suspensions of each of the twenty isolated strains were prepared in the same manner as the immunizing antigen was prepared. The original immunizing antigen was used for the parent strain in the agglutination tests.

In Table I the titers of the parent strain antiserum for each cell suspension are recorded. The tests were set up in geometric series starting at a dilution of 1 : 20. Readings were made after incubation for 12 hours at 37 C. and again after storage overnight at 4 C. No differences were observed in the two readings.

The results of these agglutination reactions indicate that strains 3 and 10 either were variants of *L. monocytogenes* which had been altered sufficiently to result in a change in the surface antigens or were grampositive, non-sporeforming, motile contaminants. Strains 19 and 21 agglutinated spontaneously in saline when agglutination reactions were attempted. Since physiological reactions did not characterize these strains as *L. monocytogenes*, each of them was tested by the precipitin reaction. Although strains 9 and 16 were agglutinated typically by

antiserum prepared against the parent strain, they were so different from the parent culture physiologically, that they were included in the precipitin reactions which were run subsequently.

For the precipitin reactions the same antiserum was used as had been used for the agglutination tests. The antigens were prepared by growing each strain in 1 per cent glucose yeast extract broth for 24 hours, after which the cells were washed twice in distilled water and suspended in physiological saline. A count was made on each suspension by the serial dilution method of McCrady (1918) and the most probable numbers were determined by consulting the tables of Hoskins (1934). Each suspension was adjusted to approximately 50 million viable cells per ml. by the addition of normal saline. These suspensions were frozen and thawed three times by placing them in the deep freeze unit overnight and allowing them to thaw out the following day. The antigens were then clarified by filtration through Seitz disks. Precipitation tests were set up by placing 0.1 ml. of the antiserum in a series of precipitin tubes and overlaying the antiserum with 0.1 ml. of various dilutions of each antigen. The controls used were: antiserum plus saline, antiserum plus 1 : 10 yeast extract broth, antiserum plus 1 : 10

TABLE I

AGGLUTINATION TITERS OF THE ANTISERUM OF THE PARENT STRAIN FOR VARIANTS OF *Listeria monocytogenes*

STRAINS	DILUTION OF ANTISERUM OF PARENT STRAIN								Saline Control
	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	
Parent, 1, 2, 5–8, 11–18, 20....	+	+	+	+	+	+	—	—	—
9.....	+	+	+	+	+	+	+	.....	.....
19, 21.....	+	+	+	+	+	+	+	+	+
3, 10.....	—	—	—	—	—	—	—	—	—

nutrient broth, and normal serum from an uninoculated rabbit plus varying dilutions of the antigen of the parent culture. Reactions were read after 3 hours at 37 C. and again after 24 hours at 7 C.

All of the controls were negative. Strains 3, 9, 16, 19 and 21 showed precipitin reactions similar to those of the parent strain. Strain 10 showed no reaction when combined in any concentration with the antiserum prepared against the parent strain. This strain could have resulted from a mutation which inhibited the production of some constituent which was an essential part of each antigenic component of the cell. But, since it could not be established that this strain was a progeny of the parent strain through antigenic or physiologic characteristics, it was not included in the subsequent studies.

Physiological reactions

Abilities to ferment carbon compounds and litmus milk, to reduce nitrate, to produce acetylmethylcarbinol and indol, and to give a positive methyl red reaction were tested with each strain in order to determine variations in physiological characteristics.

Each strain was inoculated into media containing 1 per cent of the compounds listed in Table II. These media were prepared by adding solutions of the carbon sources, which had been filtered through Seitz disks, to a 1 per cent tryptone broth adjusted to pH 7.0 and to which had been added brom cresol purple indicator. While the majority of the strains fermented the same carbohydrates, numbers 3, 8, 9, 16, 19 and 21 differed widely. Number 3 fermented mannitol and numbers

3 and 16 fermented galactose both of which the parent strain was unable to utilize. They, as well as strains 8, 9, 19 and 21, lost the ability to ferment one or more of the carbohydrates which the parent strain fermented.

Duplicate tubes of litmus milk were inoculated with each strain, incubated at 37 C, and observed at two-hour intervals for 14 hours and again after 24, 48 and 72 hours. Tubes which showed no reaction after 72 hours were observed for an additional six days but no further change was evident. The unirradiated parent strain reduced the indicator in 8 hours and produced acid in 72 hours. The two most striking variations evidenced in litmus milk were the failure to produce any observable reaction (strains 9, 16, 19 and 21) and the failure to reduce the indicator before producing acid (strains 3, 13, 15, 17 and 18).

Each strain was tested for its ability to reduce nitrates, to produce acetylmethylcarbinol and indol, and the ability to lower pH sufficiently to give a positive

TABLE II

FERMENTATION REACTIONS OF IRRADIATED STRAINS OF *Listeria monocytogenes*

Carbon Compound	Strain						
	Parent, 1, 2, 5, 6, 7, 11, 12, 13, 14, 15, 17, 18 20	3	8	9	16	19	21
Arabinose	+	+	+	+	+	+	—
Dextrin	+	—	+	+	+	+	—
Dulcitol	—	—	—	—	—	—	—
Galactose	—	+	—	—	+	—	—
Glucose	+	+	+	+	+	+	—
Inositol	—	—	—	—	—	—	—
Inulin	—	—	—	—	—	—	—
Lactose	+	—	—	—	—	—	—
Mannitol	—	+	—	—	—	—	—
Melezitose	+	—	+	—	—	—	—
Raffinose	+	+	+	+	—	+	—
Rhamnose	+	+	+	—	+	—	—
Salacin	+	+	+	—	+	—	—
Sucrose	+	—	+	+	—	+	—
Xylose	+	—	+	—	—	+	—

+ Production of acid; —, growth but no acid production.

methyl red test. Equally turbid suspensions of each strain were prepared for the inocula and the tests were carried out as prescribed in Manual of Methods for Pure Culture Study of Bacteria. All strains failed to produce indol. Ten strains produced reactions identical with those of the parent strain. Results are listed in Table III. Those strains which differed from the parent only in the production of weak Voges-Proskauer reactions all produced acid in litmus milk without preliminary reduction of indicator. Strain number 3 behaved differently from the others by producing acid in litmus milk without preliminary reduction of indicator and by showing a negative Voges-Proskauer reaction. Strain number 16 differed by showing no reaction in litmus milk, by producing a faintly positive methyl red reaction and by reducing nitrates. The other strains which showed variation all gave negative reactions in litmus milk and in the four tests indicated in Table III.

Pathogenesis

In order to determine whether any variations had occurred in the pathogenicity of these strains, they were inoculated into embryonated chick eggs. Each strain

was grown for 48 hours on blood agar slopes which were then washed down with physiological saline. These saline suspensions were adjusted to equal turbidities and varying dilutions were inoculated on the chorio-allantois of 11-day old chick embryos. Immediately following inoculation, the most probable number of viable cells in each suspension was determined by the serial dilution method. Each of five embryonated eggs was inoculated with 0.5 ml. of each dilution of each suspension.

TABLE III

ADDITIONAL BIOCHEMICAL REACTIONS OF VARIANTS OF *Listeria monocytogenes*

STRAIN	TEST			
	Nitrate	MR	VP	Indole
Parent, 1, 2, 5, 6, 7, 8, 11, 12, 14, 20	—	++++	++++	—
3, 9, 19, 21	—*	—*	—*	—*
13, 15, 17, 18	—	++++	++	—
16	++++	+	—	—

*=poor growth medium

TABLE IV

CHICK EMBRYOCIDAL ACTIVITY OF STRAINS OF *Listeria monocytogenes* IN TWO DAYS

STRAIN	MORTALITY RESULTING FROM THE INOCULATION OF INCREASING NUMBERS OF CELLS						
	5×10^2	5×10^3	5×10^4	5×10^5	5×10^6	5×10^7	5×10^8
Parent	0/10	0/10	6/10	10/10	10/10	10/10	...
1	0/5	0/5	2/5	5/5	5/5	...	...
2	1/5	3/5	5/5	5/5	5/5	...	...
3	0/5	0/5	0/5	0/5	0/5	0/5	...
5	0/5	1/5	1/5	3/5	5/5	...	...
6	1/5	2/5	5/5	5/5	...	...	...
7	2/5	3/5	5/5	5/5	...	...	...
8	0/5	3/5	4/5	5/5	...	...	...
9	0/5	0/5	0/5	0/5	...	...	...
11	0/5	0/5	1/5	2/5	5/5	...	...
12	0/5	1/5	3/5	4/5	5/5	...	...
13	0/5	2/5	2/5	3/5	5/5	...	...
14	0/5	3/5	3/5	5/5	5/5	...	...
15	0/5	1/5	1/5	3/5	5/5	...	...
16	0/5	0/5	0/5	0/5	0/5	0/5	0/5
17	0/5	0/5	2/5	3/5	5/5	...	...
18	0/5	1/5	3/5	5/5	5/5	...	...
19	0/5	0/5	0/5	0/5	0/5	...	...
20	1/5	4/5	5/5	5/5	5/5	...	...
21	0/5	0/5	0/5	0/5	0/5	0/5	...

All 19 of the possible variants showed some slight variation, but 5 strains showed a decrease in virulence which was beyond the range attributable to experimental error. These results are recorded in Table IV.

Fifty thousand viable cells of the parent strain killed three of the five embryos when first tested and two of the five when rechecked. Five hundred thousand

or more cells consistently produced 100 per cent mortality. Fifty million viable cells of strains 3, 9, 16, 19 and 21 failed to kill any embryos. Five hundred million viable cells of strain 16 produced no deaths in the chick embryos. Virulence tests of all five variants and the parent culture were repeated in embryonated eggs and the same results were observed.

Each strain which showed significant variation in virulence for the chick embryo was further tested for pathogenicity in mice. Young female white mice were injected intraperitoneally with suspensions of the variants which had been standardized previously. In some preliminary tests, the injection of thirty-nine million viable cells of the parent strain killed all of the mice within two days. To assure that the inocula contained the same or larger numbers of variant cells than were present in a lethal dose of the parent strain, a dosage of fifty million viable cells was selected as the standard inoculum.

Twenty mice were used for each strain and the results are recorded in Table V. Whereas fifty million cells of the parent strain killed all of the mice before the fifth day a similar inoculum of each of the other strains resulted in only 5–10 per cent of deaths.

TABLE V

MORTALITY IN 20 MICE INJECTED INTRAPERITONEALLY WITH 5 x 10^7 CELLS OF VARIANTS OF *Listeria monocytogenes* INDUCED WITH RADIOPHOSPHORUS

STRAIN	DEATHS ON DAY								PERCENT MORTALITY
	1	2	3	4	5	6	7	8	
Parent	4	9	6	1	0	0	0	0	100
3	0	1	0	0	0	0	0	0	5
9	0	0	0	1	0	0	0	0	5
16	1	0	0	0	0	0	0	0	5
19	1	0	0	0	0	0	0	0	5
21	2	0	0	0	0	0	0	0	10

Those mice which were inoculated with variant strains, and which survived, were reinoculated six weeks later with a lethal dose of the parent strain to determine whether they were immune. Each mouse was injected intraperitoneally with approximately 50 million viable cells. Twenty mice which had not previously been inoculated were treated similarly and they served as the controls. All of the mice died by the seventh day. This indicates that any immunity against the parent strain, which may have been conferred by the variant strains in the manner in which they were used, was of such an insignificant or transient nature as to be of little or no value for protection.

DISCUSSION

Nineteen different strains were isolated from a suspension of *Listeria monocytogenes* after exposure to beta radiation. Although these strains differed from the unirradiated parent strain in colony characteristics, all were similar in general cell morphology, in motility, and in the possession of some common antigen.

Most of the changes noted in this investigation, as in the case with most studies of induced mutations in bacteria, were changes involving loss of metabolic abilities and/or pathogenicity. In two instances, however, enzymatic activities which were either absent or completely suppressed in the parent strain were observed in the mutants; strain number 3 produced acid from mannitol and galactose and

strain number 16 fermented galactose and reduced nitrates to nitrites. It is conceivable that the apparent gain of enzymes as required by these reactions might be due to the loss of enzyme inhibitors. No information to support or refute this hypothesis was obtained in the present investigation. .

The five strains of *L. monocytogenes* which showed alteration in pathogenicity also failed to hemolyze horse blood, to ferment lactose and melezitose, and to produce strongly positive reactions in the methyl red and Voges-Proskauer tests. In addition to the changes which were common to the five strains, each of these strains varied in some manner which was not common to the others or the parent. It seems improbable that each physiologic alteration observed in this investigation was the result of a "hit" by a beta particle of a different sensitive region. A more probable mechanism might be that in several instances either a single beta particle struck a center of genetic determinants which resulted in the simultaneous alteration of several physiologic characteristics or that a single determinant was altered which alone influenced several enzyme systems.

Although administration of large inocula of the five non-virulent living cultures did not protect mice challenged six weeks later with the virulent parent culture, one should not conclude that no protection can be afforded by this means. A more detailed study with modified procedures should be made.

SUMMARY

Cells of *Listeria monocytogenes* were suspended in a medium containing radioactive phosphorus at a total radiation level of approximately 45,000 equivalent roentgens over an eleven-day period. At 24-hour intervals samples were removed and streaked on suitable media. Nineteen strains were isolated which showed variations from the parent in their colonial characteristics. Some of these also showed variations in physiological characteristics, in their antigenicity, and in their capacity to produce disease.

Most of the physiological changes which were detected were indicated by the inability of the variant to produce metabolic reactions which the parent strain was capable of producing. Two strains differed, however, in being able to produce biochemical reactions which the parent was unable to do. Five strains exhibited a greatly decreased virulence for the chick embryo and the white mouse. Heavy inocula of living cells of these strains, however, failed to protect mice when challenged with the parent strain six weeks later.

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